



Cancer stem-like cells of ovarian clear cell carcinoma are enriched in the ALDH-high population associated with an accelerated scavenging system in reactive oxygen species



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HIGHLIGHTS

- Aldehyde dehydrogenase (ALDH)1 expression is related to poor prognosis in ovarian clear cell carcinoma.
- Antioxidant enzymes are upregulated in ALDH-high cells, associated with Nrf2 induction.
- The Nrf2-antioxidant pathway might be relevant to inducing chemoresistance of CSCs in ovarian clear cell carcinoma.

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ABSTRACT

Objective. In ovarian cancer cases, recurrence after chemotherapy is frequently observed, suggesting the involvement of ovarian cancer stem-like cells (CSCs). The chemoresistance of ovarian clear cell carcinomas is particularly strong in comparison to other epithelial ovarian cancer subtypes. We investigated the relationship between a CSC marker, aldehyde dehydrogenase 1 (ALDH1), and clinical prognosis using ovarian clear cell carcinoma tissue samples. Furthermore, we investigated the antioxidant mechanism by which CSCs maintain a lower reactive oxygen species (ROS) level, which provides protection from chemotherapeutic agents.

Methods. Immunohistochemical staining was performed to examine the CSC markers (CD133, CD44, ALDH1) using ovarian clear cell carcinoma tissue samples (n = 81). Clear cell carcinoma cell lines (KOC-7C, OVTOKO) are separated into the ALDH-high and ALDH-low populations by ALDEFUOR assay and fluorescence-activated cell sorting (FACS). We compared the intracellular ROS level, mRNA level of the antioxidant enzymes and Nrf2 expression of the two populations.

Results. High ALDH1 expression levels are related to advanced stage in clear cell carcinoma cases. ALDH1 expression significantly reduced progression free survival. Other markers are not related to clinical stage and prognosis. ALDH-high cells contained a lower ROS level than ALDH-low cells. Antioxidant enzymes were upregulated in ALDH-high cells. ALDH-high cells showed increased expression of Nrf2, a key transcriptional factor of the antioxidant system.

Conclusions. ALDH-positive CSCs might have increased Nrf2-induced antioxidant scavengers, which lower ROS level relevant to chemoresistance in ovarian clear cell carcinoma.

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Introduction

Globally, epithelial ovarian cancer is the most lethal gynecological cancer. Ovarian cancer is relatively sensitive to first-line chemotherapy based on platinum/taxane [1]. It has been implied that ovarian clear cell

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Table 1
Clinical characteristics of 81 ovarian clear cell adenocarcinoma patients.

characteristics	ALDH expression		P-value
	<10%	≥10%	
Number	53	28	
Age (years)			
<50	23	7	0.17 ^a
≥50	31	21	
Median follow-up time (months)	24.5	33	0.24 ^b
FIGO stage			
I, II	23	3	0.002 ^a
III, IV	30	25	

^a P-values were determined by two side tests.

^b P-value was determined by Mann-Whitney's U test.

carcinoma has shown resistance to conventional platinum-based chemotherapy. Recent studies have shown that objective response was observed in 11–27% of patients treated with a conventional platinum-based regimen, whereas patients with the serous adenocarcinoma (SAC) subtype showed a significantly higher response rate of 73–81% [2–4]. The majority of patients will have a relapse of disease within 5 years. Recurrent disease is typically less responsive to current chemotherapeutic strategies.

The cancer stem cell model has been understood in recent cancer research. Cancer stem-like cells (CSCs) are defined as having high tumorigenicity, multiple differentiation ability and self-renewal capacity [5]. In addition, these characteristics are related to chemoresistance.

Among several markers that have been used to identify CSCs, aldehyde dehydrogenase-1A1 (ALDH1A1)-active populations have been identified as tumor-initiating cells in multiple malignancies including those of the breast [6], colon [7], pancreas [8], and liver [9]. Landen et al. were the first to isolate putative CSCs in ovarian cancer by high ALDH activity and showed that high ALDH expression predicts poor outcome in ovarian cancer patients [10]. Furthermore, Liu et al. demonstrated that elevated ALDH expression was associated with poor prognosis using meta-analysis [11].

Recently, reactive oxygen species (ROS) have been associated with multiple cellular functions such as cell proliferation, differentiation and apoptosis.

Though little is known about the ROS levels of CSCs, recent studies indicate that CSCs show lower intracellular ROS content than non-CSCs, which may be due to the increased expression of free radical scavenging systems [12,13]. Regarding CSC molecules, Kim et al. showed that CD13 negatively regulates ROS with a resultant increase of stemness in liver CSCs. CD13 is also associated with increased ROS scavenger capacity in human liver CSCs [14].

We investigated the relationship between ALDH1 expression as a CSC marker and clinical prognosis using ovarian clear cell carcinoma tissue samples. Furthermore, we analyzed the relationship between ovarian CSC and oxidative stress, by examining the involvement of the antioxidant pathway in ALDH high activity cells.

Material and methods

Patients and clinical data

We analyzed ovarian clear cell carcinoma samples from 81 patients who were diagnosed with clear cell carcinoma and undergone initial surgery at 7 hospitals in Japan (Nagoya City University Hospital, Nara Prefecture General Medical Center, Kansai Rosai Hospital, Shizuoka Cancer Center Hospital, Kurume University Hospital, Oita University Hospital, and Gifu University Hospital). These sample and patient's information were kindly provided from Kansai Clinical Oncology Group/intergroup study in Japan. We obtained relevant clinical data on patients whose tissue samples we studied by retrospective review of the patients' records. Median age is 53 years (minimum, 31; maximum, 82) and clinical stage varies from stage I (25, 30.9%), stage II (1, 1.2%), stage III (41, 50.6%) and to stage IV (14, 17.3%). Optimal primary surgeries were performed in 57 patients, and suboptimal surgeries were done with other patients.

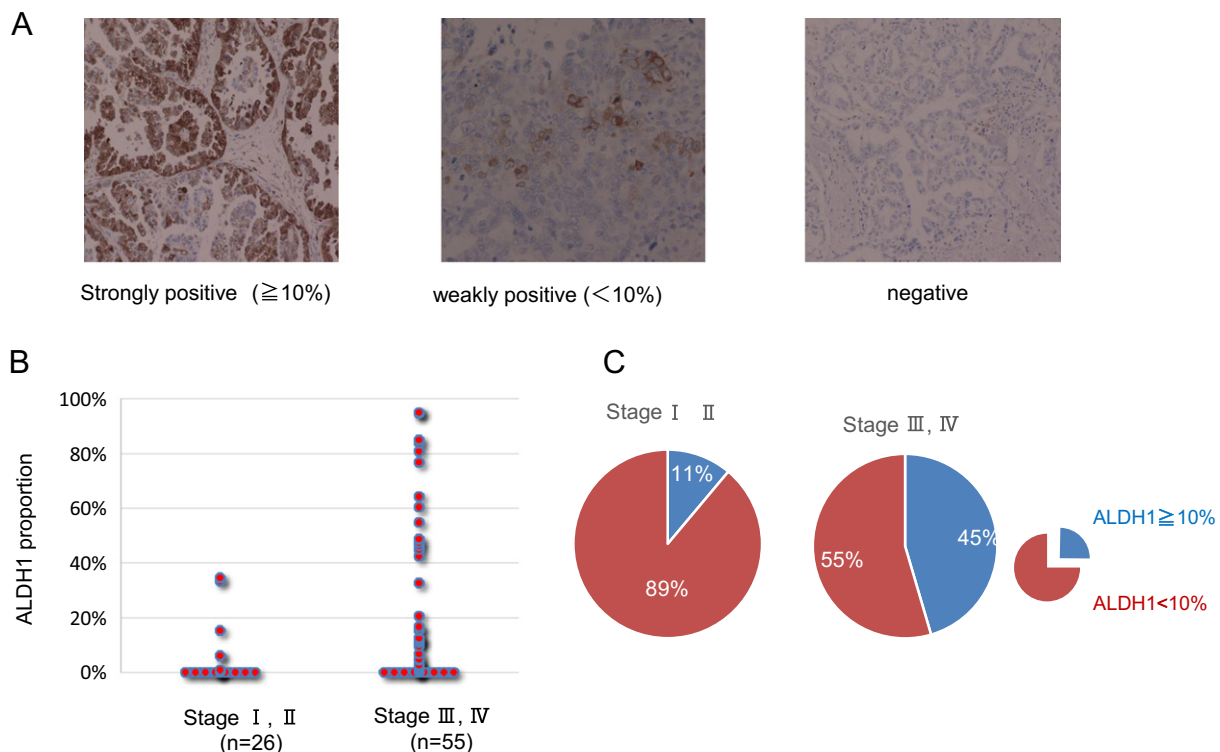


Fig. 1. Immunohistochemical analysis of ALDH1 expression in ovarian clear cell adenocarcinoma tissues. A: Strongly positive: percentage of positive cells is over 10% in the tumor tissue. Weakly positive: percentage of positive cells is between 10% and 0.1% in the tumor tissue. B and C: Proportion of ALDH1 expression in cells of different clinical stages. Percentage of strongly positive stained cells was significantly higher in advanced stage (stages III, IV, $P < 0.01$).

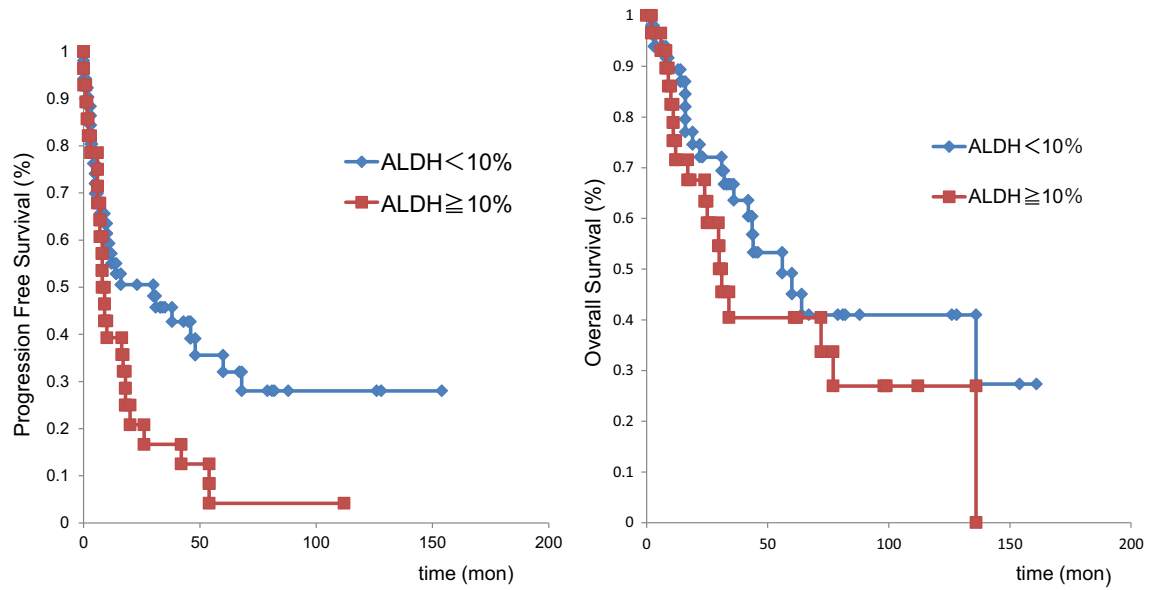


Fig. 2. Kaplan–Meier survival curves of patients with high and low levels of ALDH1 expression. A: PFS and ALDH1 expression. PFS was significantly reduced in the ALDH-high group compared with the ALDH-low group ($P < 0.05$). B: OS and ALDH1 expression. OS was not statistically related to ALDH1 expression.

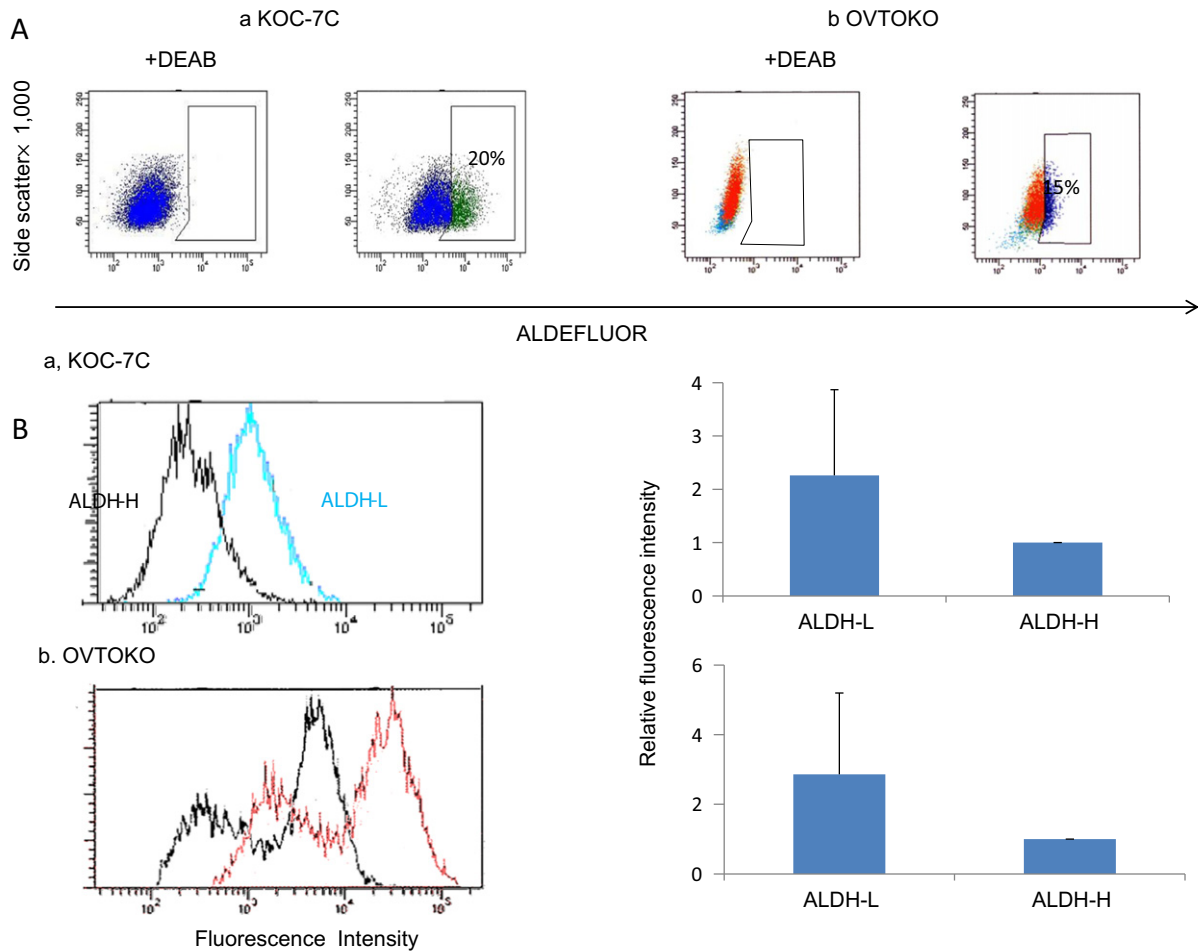


Fig. 3. ALDH activity and ROS in ovarian cancer cell lines. A: FACS analysis showing DEAB control and ALDEFLUOR activity in KOC-7C and OVTOKO. B: ALDH-high cells contained lower intracellular ROS level in ovarian clear cell carcinoma. Cells were stained with Cell-ROX. ROS levels were determined by measuring the fluorescence intensity of the cells using FACS.

Immunohistochemical staining

Immunohistochemical staining of ALDH1 was performed with formalin-fixed, paraffin-embedded section (4 μ m thick) of 81 ovarian clear cell adenocarcinoma tissues.

After deparaffinization and hydration, sections were microwaved for 20 min in 10 mM citrate buffer (pH 6.0), to unmask the epitopes. Endogenous peroxidase activity was blocked using 3% hydrogen peroxidase. The slides were then incubated overnight at 4 °C with primary mouse monoclonal antibody against ALDH1 (clone 44/ALDH, 1:750 dilution BD biosciences, San Jose, CA), CD133 (1:100 dilution Abnova, Taipei, Taiwan), CD44 (1:100 dilution BD biosciences) followed by incubation with biotinylated secondary anti-mouse IgG antibody (DAKO, Glostrup, Denmark) for 20 min, and finally with streptavidin–HRP complex (DAKO) for 20 min. Tissues were then stained for a few minutes with 3,3'-diaminobenzidine (DAKO).

The total number of cells with positive staining for antibodies was quantitated in 5 fields in each paraffin tissue section. The number of positive cells was counted on each field and the ratio against all cancer cells was calculated.

Cell lines and cell culture

In this study, two ovarian clear cell adenocarcinoma cell lines were used (KOC-7C and OVTOKO; kindly provided by Dr. H Itamochi, Tottori University, Tottori, Japan). KOC-7C cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; DAKO). OVTOKO cells were cultured in RPMI1640 (DAKO). Each medium was supplemented with 10% fetal bovine serum (FBS). Cells were incubated in a humidified 5% CO₂ incubator at 37 °C.

Nuclear extract preparation and western blotting

Nuclear extracts were prepared from the cells as previously described [15]. The protein content in the nuclear extracts was determined using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Cells were subjected to immunoblot analysis by standard techniques. Briefly, total nucleic acids were separated on 7.5% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% skimmed milk for 1 h and incubated overnight at 4 °C with anti-Nrf2 antibody (Cell Signaling, Beverly, MA) at 1:1000 dilution, or anti-Lamin B1 antibody (Bioworld Technology, St Louis, MN) at 1:1000 dilution. After washing, blots were incubated with mouse anti-rabbit IgG secondary antibody (KPL, Gaithersburg, MD). Proteins were detected using an enhanced chemiluminescence reagent (GE Healthcare, Buckinghamshire, UK).

ALDEFLUOR assay and isolation by fluorescence activated cell sorting (FACS)

ALDH activity was detected using an ALDEFLUOR assay kit (StemCell Technologies, Vancouver, Canada) according to the manufacturer's protocol. Briefly, single cells were suspended in ALDH assay buffer containing ALDH substrate BODIPY-aminoacetaldehyde (BAAA) and incubated at 37 °C for 40 min. The specific ALDH inhibitor diethylaminobenzaldehyde was used as a negative control at 50 mmol/l. Stained cells were analyzed and sorted by FACS ArialI (BD Bio-sciences).

Quantitative real-time PCR analysis (qRT-PCR)

Total RNA was isolated with NucleoSpin RNAII (Macherey-Nagel, Duren, Germany). Quantitative reverse transcriptional-polymerase

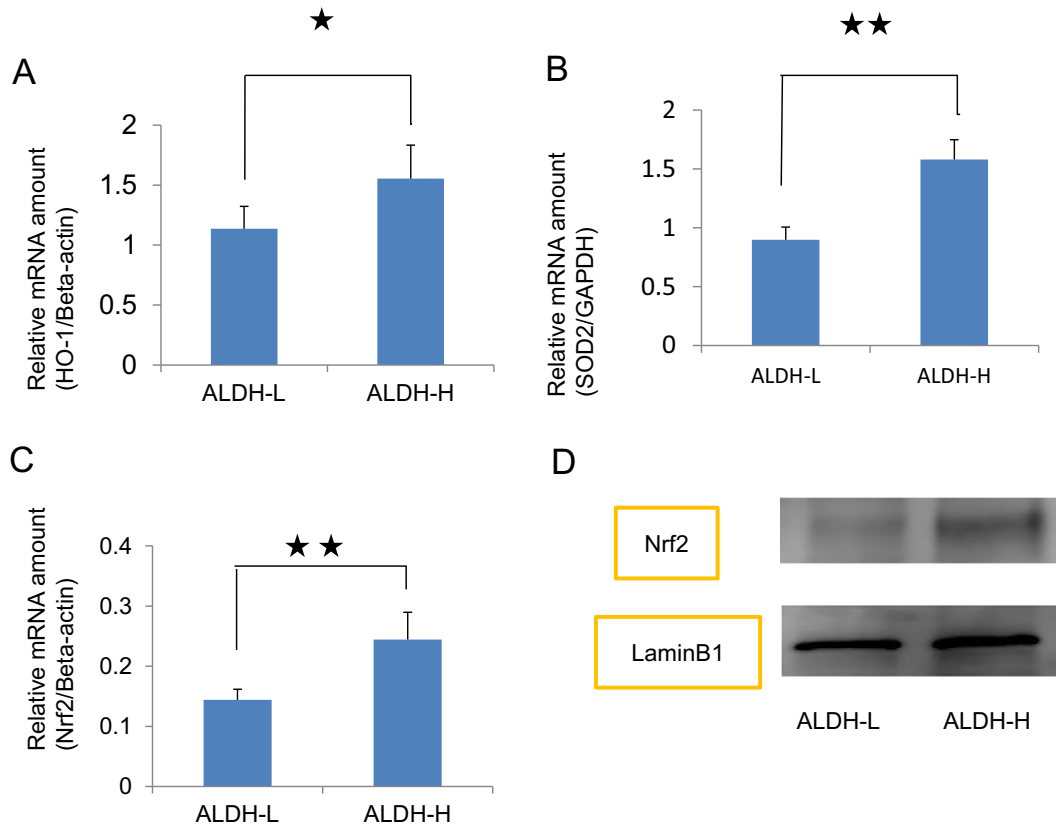


Fig. 4. Increased upregulation of antioxidant scavengers in ALDH-high cells in KOC-7C. A and B: RT-PCR analysis of relative SOD2 and HO-1 expression. Beta-actin and GAPDH were used as a reference control. C: RT-PCR analysis of relative Nrf2 expression. Beta-actin was used as a reference control. D: Protein expression of Nrf2 in nucleus fraction in ALDH-high cells and ALDH-low cells. Nrf2 protein is shown as the 110 kDa band on western blot. Lamin B1 was used as an internal control. $\star P < 0.05$, $\star\star P < 0.01$.

chain reaction (RT-PCR) was performed using a One Step SYBR Prime Script RT-PCR kit and Thermal Cycler Dice Real-Time System (TAKARA BIO Inc., Otsu, Japan). The reaction mixtures contained DNase I-treated total RNA and primers: Heme Oxygenase-1 (HO-1), sense 5'-GGAAGTTCAGAAGGGCCAGGT and antisense 5'-TGCAGCTCTTGTGGGAAGTAGAC; Nrf2, sense 5'-AGCCCAGCACATCCAGTCAG and antisense 5'-TGCATGCAGTCATCAAGTACAAAG; glutathione peroxidase 3 (GPX3), sense 5'-GACTGCCATGGTGGCATAAGTG and antisense 5'-GACCAGACCGAATGGTGCAA; and superoxide dismutase 2 (SOD2), sense 5'-CAGACCTGCCTTACDACTATGG and antisense 5'-CTCGGTGGCGTTGAGATTGTT. Thermal cycling (normalized to the beta-actin gene as an internal control) was performed using 40 cycles of 95 °C for 5 s followed by 60 °C for 30 s. Real-time PCR data were analyzed using the Multiplate RQ software (TAKARA BIO Inc.), which is able to treat multiple reference genes. Relative gene expression was determined by a comparative Ct method.

Assay for ROS staining

Suspended cells were incubated with 5 μ M of CellROX Deep Red Reagent (Invitrogen, Carlsbad, CA) at 37 °C for 30 min. ROS was activated by treatment with 100 mM H₂O₂ just before cell staining. Dead cells were stained with PI. Brightly, fluorescent cells were detected by FACS ArianI. Mean fluorescence intensity was compared between the ALDH-high and low populations.

Nrf2 transfection

pNLF1-NRF2 plasmid was purchased from Promega (Madison, WI).

Transient transfection of KOC-7C was carried out using FuGene HD (Roche Diagnostics, Indianapolis, IN) as per manufacturer's protocol. Then, we confirmed Nrf2 expression by western blotting.

Statistical analysis

Student's t-test was used to compare expression of the mRNA level of antioxidant enzyme and ROS level between the ALDH-high group and ALDH-low group. The Mann-Whitney U-test was used to compare the median follow-up time of the two groups. Kaplan-Meier survival curves were compared by log-rank test. P-values of <0.05 were considered significantly different.

Results

ALDH1 expression and patients' prognosis

Eighty-one subjects were included in this study, and divided into an ALDH1-high group (positive cells were >10% of cancer cells) and an ALDH1-low group (positive cells were <10% of cancer cells). Their clinical characteristics are summarized in Table 1. Though age and median follow-up time were not related to ALDH1 expression, FIGO stage was strongly related to ALDH1 expression. Comparison of ALDH1 expression between early stage (stages I and II) and advanced stage (stages III and IV) is shown in Fig. 1. The percentage of advanced stage ALDH1-high patients was significantly higher than that of early stage patients (11% vs 45%, $P = 0.002$) (Fig. 1). Kaplan-Meier survival curve analysis of 81 patients with clinical follow-up information showed that patients with high ALDH1 expression in their tumors had a significantly reduced progression free survival (PFS) rate in comparison to patients who had low ALDH1 expression ($P = 0.016$) (Fig. 2). Compared with ALDH1, the

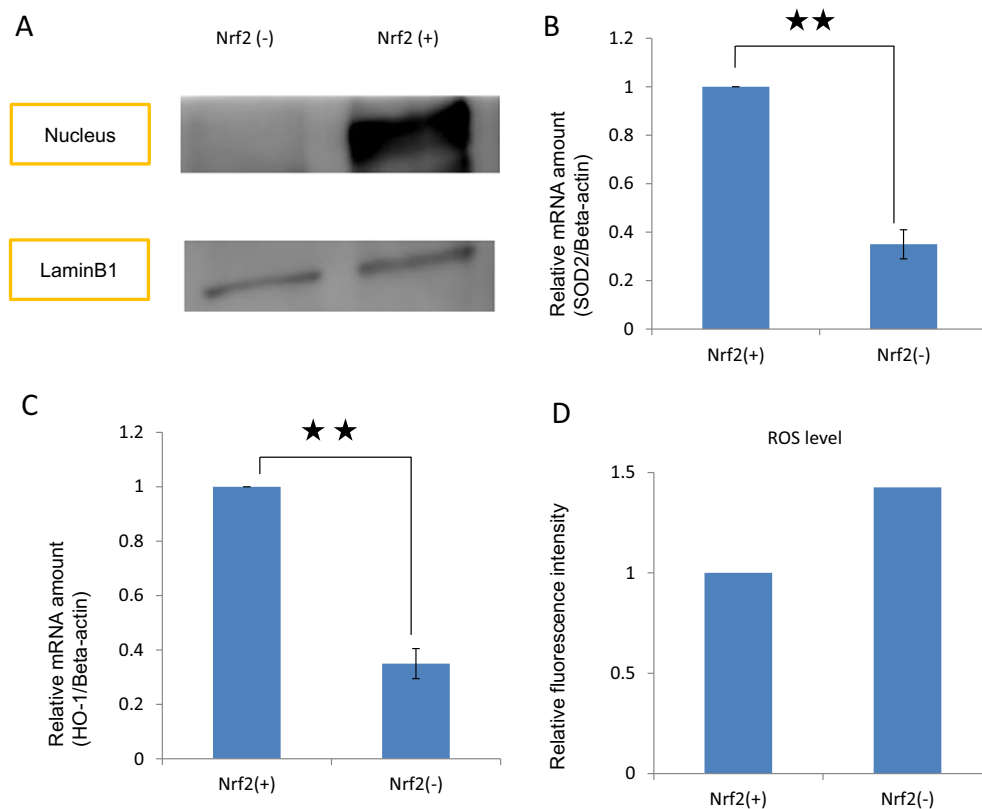


Fig. 5. Nrf2 induces antioxidant scavengers, followed by ROS reduction in KOC-7C. pNLF1-NRF2 plasmid was transfected in KOC-7C. A: Protein expression of Nrf2 in nucleus fraction was shown by western blot. B and C: RT-PCR analysis of relative SOD2 and HO-1 expression. SOD2 and HO-1 were induced by Nrf2 accumulation. Beta-actin was used as a reference control. D: ROS was inhibited in Nrf2 transfected cells. * $P < 0.05$, ** $P < 0.01$.

expressions of CD133 and CD44 were very rare. CD133 was negative in 62 (76.5%) of 81 cases. All cases were divided to CD133-high group (positive cells were >5% of cancer cells, n = 8) and CD133-low group (positive cells were <5% of cancer cells, n = 73). CD133 expression was not related to clinical stages and PFS. Furthermore, CD133 expression was not related to ALDH1 expression. In the case of CD44, almost all the samples were negative or faint by immunohistochemistry (75 of 81 cases, 92.6%). Additionally, CD44 expression was not related to clinical stages and PFS. These clinical data suggested that ALDH1 is the proper marker of CSC in ovarian clear cell carcinoma.

ALDH activity in ovarian cancer lines

Next, we analyzed the ALDH expression of cancer cell lines by FACS. We observed that the percentage of ALDH-high cells was 15–20% in KOC-7C and 10–15% in OVTOKO (Fig. 3A). The percentages of ALDH-positive cells were much lower in serous adenocarcinoma cell lines: CAOV3 was 0.1–0.3% and SKOV3 was 3–5%.

ALDH activity and intracellular ROS level

Anti-cancer agents induce the apoptosis associated with increased intracellular ROS [16]. Chemo-resistant CSCs, on the other hand, might maintain a lower ROS level. We examined the ROS levels in ALDH-high and ALDH-low cells sorted from KOC-7C and OVTOKO. The ROS level was determined by measuring the fluorescence intensity of the sorted cells using Cell ROX. ALDH-high cells had much lower intensity than ALDH-low cells in both KOC-7C and OVTOKO (Fig. 3B).

ALDH activity and antioxidant enzyme and transcriptional factor Nrf2 expression

To reveal the mechanism by which the low ROS level is maintained in ALDH-high cells, we compared the expression levels of representative antioxidant enzymes (SOD2, HO-1, GPX3) using quantitative real-time PCR. We found that SOD2 and HO-1 were significantly upregulated in ALDH-high cells in comparison to ALDH-low cells (Fig. 4A, B). However, GPX3 was not upregulated in ALDH-high cells (data not shown).

Nrf2 is known as a leucine zipper transcriptional factor, which induces antioxidant enzymes (SOD2, HO-1, GPX3) and regulates cell response to oxidative stress. Nrf2 protein was detected as a single band at 110 kDa in ovarian clear cell carcinoma cell lines by western blot. Nrf2 protein level and mRNA level in ALDH-high cells were much higher than in ALDH-low cells (Fig. 4C, D). To analyze Nrf2-mediated ROS modulation mechanism in ALDH-high cells, we need to examine the inhibition of antioxidant enzymes and ROS after knocking down Nrf2 in sorted ALDH-high cells. However, ALDH-high cells were not available for this experiment, because these sorted cells were almost dead after Nrf2-siRNA transfection. Instead, we prepared Nrf2-overexpressed cells like ALDH-high cells, and examined the induction of antioxidant enzymes and reduced ROS. Nrf2 accumulation enhanced the expression of SOD2 and HO-1, followed by ROS reduction in KOC-7C (Fig. 5).

The study provides some insight into the mechanism by which CSCs of ovarian clear cell carcinoma are associated with the Nrf2-mediated ROS reducing mechanism and may acquire resistance to chemotherapy.

Discussion

A previous study demonstrated that ALDH1-positive cells exhibit cancer stem cell properties such as self-renewal, and tumorigenicity and poor prognosis [17]. However, the prognostic significance of ALDH in ovarian cancer is controversial. Although some studies have reported that ALDH1 expression was associated with poor prognosis in ovarian cancer [17–19], Chang et al. showed that ALDH1 was a favorable predictor in ovarian cancer [20]. In addition, Ricci et al. showed that ALDH1 is not associated with prognosis [21]. Most studies include

heterologous histological types and clinical stages. Because ovarian cancer is composed of various histological subtypes and oncogenic genes differ in each subtype [32], there might not be a common CSC marker. In this study, ALDH1 expression was found to be associated with shorter PFS in ovarian clear cell carcinoma.

In general, PFS and OS are thought to be the appropriate endpoints in clinical trial. Some reports described that PFS is a surrogate for OS in advanced cancer [22,23]. However, in some diseases, an improvement of PFS does not necessarily correlate with an improved OS. Broglio et al. noted that OS is a reasonable primary endpoint when median survival post-progression (SPP) is short, perhaps less than 6 months, but is too high a hurdle when median SPP is longer than 12 months [22,24]. This scenario might also be applicable in ovarian cancer. In this study, we confirmed a statistical difference between the ALDH1-high group and ALDH1-low group in PFS, but not in OS. Given that the median SPP is long (13 months in these cases), PFS can be considered a more reasonable endpoint than OS in the present study. ALDH1 expression is therefore associated with poor prognosis and might be a candidate CSC in ovarian clear cell carcinoma. However, the multivariate analysis including stage and optimal/suboptimal surgery, revealed that ALDH1 positivity is a marginal significant prognostic factor in these clear cell carcinoma cases ($P = 0.07$, Cox proportional hazard's model).

Commonly used chemotherapeutic regimens induce high levels of ROS that kill cancer cells [25]. Diehn et al. reported that breast CSCs contain a lower ROS level and enhanced ROS-scavenging systems in comparison to non-CSCs [12]. In addition, lower ROS levels are associated with less DNA damage by ionizing irradiation. Antioxidant enzymes are upregulated in human cancer cells or tissues and may contribute to chemoresistance [26]. The antioxidant system of cancer cells might therefore be relevant to cell survival against anti-cancer therapies.

In this study, we have revealed that ALDH-high cells had a lower ROS level than ALDH-low cells and suggested the elevated antioxidant enzyme.

As described above, ovarian clear cell carcinoma shows resistance to ovarian cancer-standardized platinum-based chemotherapy. Platinum compounds form electrophilic intermediates that mediate DNA cross-linking and induce double-strand DNA breaks. Cellular response to electrophilic xenobiotics is partly mediated by the Nrf2-Keap1 antioxidant pathway [27]. Nrf2, a leucine zipper transcriptional factor, regulates cell response to oxidative stress. Nrf2 target genes are mainly antioxidant enzymes such as SOD2, GPX and HO-1 [16,28]. Superoxide (O_2^-), the principal form of ROS, is rapidly converted to hydrogen peroxide (H_2O_2) by SODs. H_2O_2 is then converted to H_2O and O_2 by GPX through coupling with the transduction of glutathione (GSH) to oxidized GSH. Yeung et al. showed that the knockdown of SOD2 by siRNA leads to the sensitization of ovarian cancer cells to the anticancer agents doxorubicin and paclitaxel, whose action involves the generation of free radicals [29]. Saga et al. reported that GPX3 suppression by siRNA increased cisplatin sensitivity in ovarian clear cell carcinoma [30].

Additionally, HO-1 is an enzyme responsible for degradation of heme into free iron, carbon monoxide, and biliverdin. These end-products have antioxidant activities [31]. We observed that SOD2 and HO-1 were induced in ALDH-high cells without any increase of GPX3. We could not confirm elevated GPX3 level in ALDH-high cells in two cell lines (data not shown). The elevated antioxidant enzymes may vary in each of the tissues and cell lines.

To our knowledge, this is the first report describing the Nrf2-antioxidant mechanism in CSCs of ovarian cancer. Further investigation to reveal the antioxidant mechanism might overcome the chemoresistance of ovarian clear cell carcinoma.

Conflict of interest statement

The authors state that there are no potential conflicts of interest.

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